Tissue-Specific Markers in Flow Cytometry of Urological Cancers. III. Comparing Chromosomal and Flow Cytometric DNA Analysis of Bladder Tumors


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Thirty-seven transitional-cell carcinomas (TCC) of the urinary bladder were analyzed by DNA flow cytometry (FCM). After labelling of the cell suspensions with antibodies to cytokeratin, the cytokeratin-positive cells and the non-epithelial cytokeratin-negative cells could be analyzed separately. After estimation of S- and G1M phase, 3:17 cases (18%) with a normal DNA index showed elevated proliferative levels, among cytokeratin-labelled suspensions only. Of these 17 cases, 14 showed chromosomal abnormalities. The remaining 20 cases with abnormal, irrespective of the technique used. All, immuno-labelling of tumor cells for cytokeratin in FCM increases the sensitivity of this method in detecting aneuploid tumors or tumors with high proliferation fractions, the discriminating power of chromosomal analysis of TCC is greater than FCM.

Chromosomal analysis as well as DNA flow cytometry have been described as important methods for studying the biology of urinary bladder cancers (Granberg-Ohman et al., 1980, 1982, 1984; Wijkström et al., 1984). An increase in chromosome number has been reported to correlate with invasiveness and loss of differentiation (Granberg-Ohman et al., 1980; Lamb, 1967; Spooner and Cooper, 1972). Furthermore, marker chromosomes are regarded as characteristics of clinically more aggressive bladder tumors (Palor and Ward, 1978; Sandberg, 1980, 1982; Summers et al., 1981).

DNA measurements of transitional-cell carcinoma (TCC) showed that an abnormal DNA value is a sign of malignancy (Levi et al., 1969; Collste et al., 1980; Tribukait et al., 1982; Barlogie et al., 1983; Frankfurt et al., 1984). DNA values obtained through FCM and proliferative data are useful tools for diagnosis, management and prognosis of patients with bladder tumors (Devoucex et al., 1981; Gustafson et al., 1982; Klein et al., 1982; Chin et al., 1985). Normal modal chromosome number and DNA index obtained by FCM are in good agreement with each other in TCC. However, tumors with near diploid chromosome counts and pseudo-diploid abnormalities can apparently not be detected by FCM (Granberg-Ohman et al., 1980; Wijkström et al., 1984). This may be due to the fact that the DNA FCM analysis of carcinomas is often disturbed by the presence of stromal or inflammatory cells in the cell suspensions obtained from these neoplasms. Recently the use of antibodies to cytokeratin and FCM has made it possible to distinguish between the different tissue types present in such suspensions (Ramaekers et al., 1983, 1984). Virtually all carcinomas, including TCC, contain cytokeratins, contrary to diploid stromal and inflammatory cells. Therefore, immuno-labelling of tumor-cell suspensions with cytokeratin antibodies makes possible the analysis of epithelial cells by FCM (Feitz et al., 1985). In this way, the proliferative fraction among tumor cells can be estimated apart from stromal and inflammatory cells. This method allows cytokeratin-negative diploid cells to be used as internal standards for calculation of the DI values of the tumor cells. This cannot be done without this labelling procedure, since in that case diploid carcinoma cells cannot be distinguished from non-epithelial cells.

In this study, data on chromosomal analysis and on FCM DNA measurements in tissue specimens from patients with TCC of the urinary bladder, with and without application of antiseria to cytokeratin, were compared. The proportion of tumor cells in S-phase, estimated after application of these antiseria, was correlated with tumor stage.

Material and Methods

Thirty-seven tumor specimens, 33 primary and 4 recurrent cases, from 26 male and 7 female patients with TCC of the urinary bladder were successfully analyzed. According to tumor stage, they could be divided as follows: 14 cases of pTa, 11 of pT1 and 12 of pT2-T4. The tissues were collected following transurethral resection. One part of each biopsy specimen was used for pathologic examinations, while the rest was used for chromosomal and flow cytometric studies with and without application of antiseria against cytokeratin.

Clinical staging of the tumor was done according to the UICC TNM system (Harmer, 1978) and grading according to the WHO system (WHO, 1973).

Chromosomal analysis

One or both of the following techniques were used: Direct method. The tissue specimens were collected and transported in 10 ml 0.5% sodium citrate + 0.5 μg Colcemid/ml (total time in solution: 1 hr). They were disaggregated by scraping and cutting in a Petri dish and filtered through a 100-μ nylon filter (Ortho, Bence, Belgium). After incubation in Hank's balanced salt solution (BSS obtained from Gibco, Paisley, UK), Colcemid was added (2 μg/ml Hank's BSS) for 30 min. Hypotonic treatment was done with a mixture of 6 ml fetal calf serum (FCS, Gibco) and 24 ml 0.052 M KCl. Fixation was performed with acetic acid-methanol 3:7.

Short-term culture (Smeets et al., 1985). The tissue specimens were collected and transported in 10 ml RPMI 1640 medium plus 17% FCS, 50 μg gentamycin/ml, 50 U penicillin/ml and 50 U streptomycin/ml. They were disaggregated mechanically, then incubated for 24 hr in the medium mentioned above. Metaphase arrest with Colcemid (0.1 μg/ml) was followed by hypotonic treatment with 0.075 M KCl and fixation in methanol-acetic acid 3:1.

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All slides were stained with Giemsa.

C-banding and G-banding were performed (if sufficient appropriate metaphases were available) and metaphases photographed and analyzed according to the standard rules (ICSN, 1978).

Chromosomal range, modal number, distribution according to ploidy (n=23±11, 2n=46±11, 3n=69±11 etc.) and the presence of marker chromosome(s) were registered. Results were pooled in those cases where metaphases were obtained with the direct method as well as with the short-term culture method.

Flow cytometry

The specimens were collected in 10 ml RPMI 1640 medium plus 17% FCS, 50 μg gentamycin/ml, 50 U penicillin/ml and 50 U streptomycin/ml. The cell suspensions were prepared mechanically as described above for the suspensions used for chromosomal analysis. The filtered cell suspensions were centrifuged at 400 g for 7 min. Then 70% ethanol (−20°C) was added rapidly to the cell pellet with constant shaking. The final concentration was about 3 × 10^6 cells/ml ethanol. At this stage, the fixed cells could be stored for several weeks at −20°C.

Immunohistochemical cell staining procedures. Cells were incubated with a polyclonal antibody to cytokeratin (pKer; Eurodiagnostics, Apeldoorn, The Netherlands) for an indirect immunofluorescence technique (Ramaekers et al., 1983). About 1 × 10^5 cells in 70% ethanol were centrifuged (400 g; 1.5 min) and the pellet was resuspended with 1 ml of 5% FCS in buffered A (8.01 g NaCl, 0.2 g KCl, 1.44 g NaHPO4, 0.2g KH2PO4 in 1,000 ml water, pH 7.4) and pelleted again. The cell pellet was resuspended in 0.2 ml of the pKer antigenum, diluted 1:5 in 5% FCS in buffered A. After 30 min at room temperature with regular shaking, then the cells were washed 3 times in 1 ml buffered A containing 5% FCS. After the last washing step the cell pellet was resuspended in 0.5 ml of FITC-conjugated goat-anti-rabbit IgG, diluted 1:25 (Nordic, Tilburg, The Netherlands). After incubation for 30 min with this secondary antibody, the cells were washed again.

The cell pellet was then resuspended in 1 ml of a propidium iodide (PI) solution (20 mg/ml A-grade A-grade) in 150 mM sodium phosphate buffer, pH 7.4; Calbiochem-Boehringer, La Jolla, CA). To 1 ml of this cell suspension 0.1 ml of a stock solution of RNase (1% type A in the sodium phosphate buffer; Sigma, St. Louis, MO) was added, then the cell suspension was incubated for 10 min at 37°C. Finally, the cell suspension was filtered through a 100-μ filter and the cells were kept in the dark at room temperature prior to FCM analysis (Ramaekers et al., 1984; Feitz et al., 1985).

DNA measurements. Cell analysis was performed using a Cytofluorograph 50H (Ortho, Westwood, MA). The fluorochromes PI and FITC were excited at 488 nm with an argon ion laser (Spectra Physics, Mountain View, CA). Fluorescence was measured simultaneously using a 515- to 530-nm band pass filter and a 630-nm long pass filter for FITC and PI, respectively. A correction was made for leakage of FITC fluorescence into the PI channel. All data were stored in list mode in a PDP 11/34 computer (Digital, Marlboro, MA) for subsequent data analysis. Ploidy measurements of the unlabelled tumour cells were done (Jakobsen, 1983). The DNA content was expressed as DNA index (DI) (Hiddemann et al., 1984).

The DI of the tumor cells labelled with pKer was estimated using the non-pKer-labelled cells as internal standards. The DI of normal bladder cells was 1.01 (range 0.95-1.07) with an SD = 0.04.

Samples with a DI of more than 1.15 or less than 0.89 (= mean ± 3 SD of measurements of normal bladder cells) were classified as abnormal (Smeets et al., 1987).

Percentages of cells in S- and G2-M phases were measured (Baisch et al., 1975, 1982).

The mean coefficient of variation (c.v.) for the G0/G1 peak of the tumor cells was 5.8 (range 3.5 - 9.3).

RESULTS

Chromosomal abnormalities were observed in 34 out of 37 cases that were analyzed successfully (92%). Both unlabelled as labelled in 20 cases the DI was abnormal and 17 specimens showed a normal DI. In 31/37 tumours which did not contain excessive debris, the percentage of cytokeratin-positive cells in S- and G2-M phase could be calculated (Fig. 1) which was not possible without pKer labelling. Among the 17 tumours with a normal DI, in one case the S-phase and in 2 cases the G2-M-phase were conspicuously high.

Thus, in 3 of the 17 cases, formerly thought to have a normal DNA content, application of cytokeratin antibodies gave indications of abnormalities which were not seen without such labelling.

pTa tumors

The DI of the G0/G1 fraction in 13/14 cases of superficially growing bladder tumors was normal (2e), irrespective of the labelling for cytokeratin (Table I). In one case (number 13) the DI was highly abnormal and after labelling a high percentage of cells was found in G - phase (mean 4.7 with an SD = 4.1). In one diploid tumor (5) the S-phase percentage and in another case (9) the G2-M-phase percentage were very high. Without labelling the S-fractions of the pTa tumors were not abnormal. In 11/14 cases chromosomal abnormalities were observed. With one hyperdiploid exception the modal chromosome number of these cyogenetically abnormal tumors was (hyper)-diploid, while 3 cases (5, 8 and 13) showed a wide range in their chromosome number. Marker chromosomes of different types could be obtained in 7 cases. A G-banded karyotype with some abnormal chromosomes is shown in Figure 2.

pT1 tumors

The DI of the G0/G1 fraction of 7/11 cases was abnormal, either with or without labelling. The mean percentage of cells in S-phase was 10.5 (SD = 4.1). In one case with a normal DI (8) the percentage of cells in G2-M phase was extremely high.

All cases showed chromosomal abnormalities. The modal chromosome number was (hyper)-diploid in 4 cases and hyperdiploid in 7 specimens. Marker chromosomes of different types were found in 6/11 cases.

pT2-pT4 tumors

The DI of the G0/G1 fraction of all these cases of deeply infiltrating tumors was abnormal either with or without labelling. In one case (31) 2 different cell populations with an abnormal DI were found.

The mean percentage of cells in S-phase was 13.1 (SD = 2.6). All 12 cases showed chromosomal abnormalities. The modal chromosome number was diploid in 2 cases and hyperdiploid in 12 specimens. Marker chromosomes of different types were found in 9/12 cases.

Correlation between flow cytometric and chromosomal data

The mean value of the DNA index, as determined after labelling of pTa tumors, was 6% above the value expected from the corresponding chromosome count. For pT1 tumors this was 12% and for pT2-pT4 tumors 18% (Fig. 3).
Figure 1—PCM analysis of a transitional bladder cell carcinoma after labelling for cytokeratin (pKer) and staining of DNA with P.I. (a) Two-parameter analysis, showing position of the window containing epithelial cytokeratin-positive tumor cells. (b) DNA histogram of the total cell suspension. (c) DNA histogram of the cytokeratin-positive cells, selected by placing a window as illustrated in (a). (d) DNA histogram of cytokeratin-negative cells.

Figure 2—G-banded karyotype of a Ta tumor. Note the presence of abnormal chromosomes indicated by arrows. This tumor was not found to be abnormal by FCM.

Figure 3—Correlation between the DNA index of cytokeratin-positive cells and modal chromosome number. □ = pT1 tumor, × = pT1 tumor, ○ = pT2-pT4 tumors.
TABLE 1 - RESULTS OF DNA FLOW CYTOMETRY AND CHROMOSOMAL ANALYSES OF BLADDER TRANSITIONAL-CELL CARCINOMAS IN RELATION TO TUMOR STAGE

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1DU: DNA index of the G2/M fraction of the tumor cells, estimated without labelling with pKer. 2DU: DNA index of the G1/G2 fraction of the tumor cells after labelling with pKer. 3S: Marker chromosome(s). 4S: % cells in S-phase after labelling with pKer. 5G2M: % cells in G2/M phase after labelling with pKer.

Correlation between DNA index, proliferative fraction and chromosomal number with tumor stage

The different parameters estimated from the flow cytometric studies and obtained from chromosomal analysis (Table I) were correlated with tumor stage. It appeared that DNA index, S-phase and modal chromosome number showed a strong correlation with tumor invasiveness (Figs. 4-6). Strikingly, however, 2 cases of pTa tumors (cases 5 and 13) could clearly be distinguished in these comparative studies, since they showed high values for one or more parameters. Similarly, some cases of pT1 tumors had low values less than average.

DISCUSSION

DNA flow cytometric (FCM) analysis of carcinoma cells has been described as a useful and reproducible method in diagnosis and management of patients with bladder tumors (Devonec et al., 1981; Wijkström et al., 1984). In order to analyze the carcinoma cells separately from contaminating non-epithelial cells, antibodies to cytokeratin can be used in a 2-parameter analysis (Ramaekers et al., 1983, 1984; Feitz et al., 1985, 1986). Although chromosomal analysis of bladder cancer is hampered by practical difficulties, essential information such as range in chromosome counts, marker chromosomes and the association of some chromosomal changes and bladder cancer can be obtained (Atkin and Baker, 1977, 1985; Gibas et al., 1984; Sandberg, 1984; Smeets et al., 1985; Vanni et al., 1985; Pauwels et al., 1987).

In this study, the data obtained by 2 methods of DNA analysis of cells recognized with antibodies to cytokeratin and chromosomal analysis have been compared.

The advantages of using cytokeratin antiserum have been demonstrated (Feitz et al., 1985). This study revealed 17 tumors with an apparent diploid DNA index. But, after labelling with the cytokeratin antiserum, 3 of these cases showed strikingly high percentages of cells in S- and G2/M phase. This is of importance, since high S-phase may be interpreted as an adverse prognostic factor (Barlogie et al., 1983). The high percentage of cells in G2/M phase may possibly indicate the presence of a tetraploid cell population in the tumor. Chromosomal analysis did not show any tetraploid cells in the cases mentioned. However, there is always a risk of missing some cells by cytogenetic examination, especially in specimens with low mitotic activity.

In bladder cancer cases it is of extreme prognostic and therapeutic importance to be able to distinguish between pTa...
and pT1 tumors and to recognize potentially invasive pTa tumors. Of the 17 cases of pTa with a normal DI, 3 showed indications of abnormalities when S- and G2M fractions were estimated after labelling, which thus improved the discriminatory power of FCM by 18%.

The two cases of pTa tumors with a high DI and/or S-phase are particularly interesting in this respect. These tumors did also show significant chromosome abnormalities and, therefore, should be considered potentially more aggressive cases.

Chromosomal abnormalities were present in 14/17 cases with a normal DI, while 11/17 cases showed marker chromosomes. The 20 tumors with an abnormal DI showed, within a 10% variation, the same DNA value with or without labelling for cytokeratin. All these cases showed chromosomal abnormalities.

The fact that the DI is above the values expected from the corresponding chromosome count (in this study 6-18%) has been commented earlier (Granberg-Öhman et al., 1982; Wijkström et al., 1984).

Neither premature chromosome condensation (Barlogie et al., 1983) nor an increase in the number of large chromosomes, as present in many tumors (Tribukait et al., 1986), can explain this difference. Contribution of double-stranded RNA is excluded since an RNase treatment was applied before flow cytometric analysis of the cell suspensions. It is possible that a random loss of chromosomes, especially in the strongly hyperdiploid tumors, may contribute to this difference.

The wide range of chromosome numbers, seen in non-infiltrating as well as in infiltrating tumors, is the result of a great heterogeneity and indicates a more aggressive behavior of the tumor (Pauwels et al., 1987). Banding of chromosomes is still problematic. C-banding could be done in most cases, but G-banding, which is necessary for karyotyping and identification of structurally abnormal chromosomes, could only be achieved in few cases. Nevertheless, the presence of chromosomal abnormalities was unequivocal in most instances.

Although labelling for cytokeratin improved the value of FCM analysis, the discriminatory power of chromosomal analysis appeared greater than that of FCM. Near-diploidy, marker chromosomes and wide range in chromosome count which is present in many superficial tumors, could be not recognized by FCM in most cases.
We thus suggest a combination of chromosomal analysis and flow cytometric estimation of DI, S- and G2M phase following prior labelling with antibodies to cytokeratin, when trying to recognize the potentially invasive non-infiltrating tumors.

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